PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of

Noboru YAMAJI, et al.

Appln. No.: 10/525,015

Confirmation No.: 5025

Filed: February 17, 2005

Docket No: Q86324

Group Art Unit: 1654

Examiner: Andrew D. KOSAR

For: AN AGENT FOR INHIBITING ARTICULAR CARTILAGE EXTRACELLULAR MATRIX DEGRADATION

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the provisions of 37 C.F.R. § 41.37, Appellant submits the following:

Table of Contents

I. REAL PARTY IN INTEREST

The real party in interest is Astellas Pharma Inc.

II. RELATED APPEALS AND INTERFERENCES

Appellants, Appellants' legal representative and the Assignee of this application are not aware of any other appeals or interferences which may be related to, directly affect or be affected by, or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 18-21 are pending in the application.

This is an appeal from the Examiner's rejection of claims 18-21 under 35 U.S.C. § 102(b) over Watkins (WO 02/30879 A2).

IV. STATUS OF AMENDMENTS

The Amendment submitted on August 6, 2008 is the last response submitted with amendments to the claims of the application. The Amendment filed on August 6, 2008 was entered according to the Advisory Action mailed August 19, 2008.

There are no outstanding amendments to the claims or to the specification in the present application.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention relates to a method for treating osteoarthritis caused by articular cartilage extracellular matrix degradation.

Independent claim 18 of the present application recites a method for treating osteoarthritis caused by articular cartilage extracellular matrix degradation, which comprises administering a therapeutically effective amount of a histone deactylase-inhibiting compound to a patient in need thereof. See specification page 10, lines 16-23 and page 11, lines 8-12.

Claims 19-21 ultimately depend from claim 18.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 18-21 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Watkins (WO 02/30879 A2).

VII. ARGUMENT

The rejection of claims 18-21 under 35 U.S.C. § 102(b) based on Watkins should be reversed because Watkins does not contain an enabling disclosure of the treatment of osteoarthritis; and therefore the present invention is not anticipated.

The Examiner relies on Watkins as teaching that HDAC inhibitors are well known for treating osteoarthritis.

Appellants respectfully traverse the rejection and submit that Watkins is primarily directed to inhibition of proliferative conditions such as cancer and psoriasis. Watkins only mentions osteoarthritis as a disease or condition for which the disclosed HDAC inhibitors might be useful, but it does not teach a specific working example where an HDAC inhibitor is administered to a subject actually having osteoarthritis. Thus, Watkins does not identically disclose all elements of the present claims and for at least this reason does not anticipate the present claims.

Additionally, the disclosure of Watkins et al is not enabling as to a method of treatment of osteoarthritis when taken in view of the knowledge and skill available and the state of the art. "A reference contains an 'enabling disclosure' if the public was in possession of the claimed invention before the date of invention. 'Such possession is effected if one of ordinary skill in the art could have combined the publication's description of the invention with his [or her] own knowledge to make the claimed invention.' *In re Donohue*, 766 F.2d 531, 226 USPQ 619 (Fed. Cir. 1985)." See MPEP §2121.01. The disclosure in an assertedly anticipating reference must

provide an enabling disclosure of the desired subject matter; mere naming or description of the subject matter is insufficient, if it cannot be produced without undue experimentation. *Id.*

At pages 110-111, Watkins et al states that inflammatory diseases such as osteoarthritis and rheumatoid arthritis are conditions which are known to be mediated by HDAC or are known to be treated by HDAC inhibitors. However, this statement is contrary to the knowledge and skill in the art with respect to treatment of osteoarthritis and cannot serve as an enabling disclosure.

Specifically, at pages 110-111, Watkins discloses:

> The compounds of the present invention may also be used in the treatment of conditions which are known to be mediated by HDAC, or which are known to be treated by HDAC inhibitors (such as, e.g., trichostatin A). Examples of such conditions include, but are not limited to the following:
> Cancer (see, e.g., Vigushin et al., 2001).
> Inflammatory disease (e.g., osteoarthritis, rheumatoid arthritis) (see, e.g., Dangond et al., 1998; Takahashi et al., 1996).

The mere disclosure of Watkins at pages 110-11 relied on by the Examiner is not sufficiently enabling for the treatment of osteoarthritis for purposes of anticipation under 35 U.S.C. § 102 since it does not provide any examples, direction or guidance for use of a specific HDAC inhibitor agent in the treatment of osteoarthritis. Additionally, the references cited by Watkins in support of the assertion that HDAC inhibitors were known to treat inflammatory diseases such as osteoarthritis and rheumatic arthritis do not even mention these conditions and thus do not support the assertion made by Watkins.

Specifically, Watkins is not enabling for the use of HDAC inhibitor compounds in the treatment of osteoarthritis. Watkins discloses carbamic acid compounds and teaches that the compounds are useful as HDAC inhibitors, in particular, useful to inhibit proliferative conditions, such as cancer and psoriasis. The biological activity concretely disclosed in Watkins is merely a finding of "the ability to inhibit deacetylase activity and to inhibit cell proliferation" (cf. pages 230-247). Although Watkins describes reasons for usefulness for inhibiting proliferative conditions, such as cancer and psoriasis, in detail, the description about other uses is limited to the description at pages 110-111. At page 110, lines 15-18, it is described, "The compounds of the present invention may also be used in the treatment of conditions which are known to be mediated by HDAC, or which are known to be treated by HDAC inhibitors". But Watkins does not provide any additional guidance as to how the compounds can be used to treat osteoarthritis other than the reference to Dangond et al and Takahashi et al (of record, submitted as Attachments 1 and 2 to the Amendment filed January 31, 2008) and these references do not mention osteoarthritis.

Dangond et al and Takahashi et al do not have any disclosure relating to the use of the compounds described therein in the treatment of osteoarthritis and inflammatory disease. Dangond et al describes that "HDACs suggests they play a fundamental role in multiple and complex cellular pathways of immune system regulation" and Takahashi et al discloses that Trichostatin A inhibits IL-2 gene expression and has immunosuppressive activity and proliferation inhibiting activity. Thus, it is apparent that these disclosures do not establish a nexus between HDACs and osteoarthritis. Appellants have pointed out that osteoarthritis is not a

disease relating to the immune system. Osteoarthritis is a disease where degradation of extracellular matrix constituted from collagen and proteoglycan occurs. However, as is apparent from the description of classification of autoimmune diseases (refer to Table 2-2, page 29 of Reference Document 3, of record, submitted with the Amendment filed November 27, 2006) and its mechanism of action (refer to Fig. 4, page 32 of Reference Document 4, of record, submitted with the Amendment filed November 27, 2006), osteoarthritis is not an autoimmune disease.

Also, of all the references of record in the present application, there is no reference which shows that osteoarthritis is "known to be mediated by HDAC" or "known to be treated by HDAC inhibitors". Accordingly, Watkins does not show enablement for the use of HDAC inhibitor compound in the treatment of osteoarthritis.

The Examiner has not pointed to any other references in support of his position that Watkins is enabling for the treatment of osteoarthritis. In the Advisory Action dated July 3, 2008, the Examiner states that one need not look beyond the disclosure of Watkins since Watkins states that it is well known to treat osteoarthritis with HDAC inhibitors. More specifically, the Examiner states "Watkins provides the disease, the statement it can be treated with HDACi and provides a myriad of HDACi's [*sic*] to use". However, this is not the standard for determining whether a reference is enabling. As noted above, a reference contains an enabling disclosure if the public was in possession of the claimed invention before the date of invention. Such possession is effected if one of ordinary skill in the art could have combined the publication's description of the invention with his [or her] own knowledge to make the claimed invention. See MPEP §2121.01 citing *In re Donohue*, 766 F.2d 531, 226 USPQ 619 (Fed. Cir. 1985). In this

case, the description in Watkins is insufficient and the knowledge and remainder of the prior art does not support the description in the Watkins reference that it was well known that HDACi's could be used for the treatment of osteoarthritis.

As explained above, Dangond et al and Takahashi et al, which are cited in Watkins as references for inflammatory disease (e.g., osteoarthritis, rheumatoid arthritis), describe that the immunosuppressive activity is the base. Accordingly, each of these references indicates that the HDAC inhibitor acts on rheumatoid arthritis through immunosuppressive activity.

On the other hand, as described above, osteoarthritis is neither an autoimmune disease nor a disease in which CD154, IL-10, and INF-gamma participate. Accordingly, considering the functional mechanism of the HDAC inhibitor disclosed in the references, it is quite apparent that the use for osteoarthritis would be distinguished from the use for rheumatoid arthritis by those of ordinary skill in the art.

Moreover, even if Watkins could be considered as describing osteoarthritis and rheumatoid arthritis in parallel, one skilled in the art who understood the contents of all of these references would not consider that the HDAC inhibitor can act on osteoarthritis through its immunosuppressive activity and rather would doubt its enablement. Thus, one skilled in the art would not consider to apply the HDAC inhibitor to osteoarthritis similar to rheumatoid arthritis.

In view of the above, Appellants respectfully submit that the totality of the evidence provided on the record must be considered, which establishes that Watkins is not an enabling reference and does not disclose, teach or suggest the present invention of treating osteoarthritis caused by articular cartilage extracellular matrix degradation, which comprises administering a

therapeutically effective amount of a histone deacetylase-inhibiting compound to a patient in need thereof as recited in independent claim 18. The ultimate determination of patentability is based on the entire record, by a preponderance of evidence, with due consideration to the persuasiveness of any arguments and any secondary evidence. See MPEP § 2142 citing *In re Oetiker*, 977 F.2d 1443, 24 USPQ2d 1443 (Fed. Cir. 1992). The legal standard of "a preponderance of evidence" requires the evidence to be more convincing than the evidence which is offered in opposition to it. When an Applicant submits evidence, whether in the specification as originally filed or in reply to a rejection, the Examiner must reconsider the patentability of the claimed invention and the decision on patentability must be made based upon consideration of all the evidence, including the evidence submitted by the Examiner and the evidence submitted by the Applicant. A decision to make or maintain a rejection in the face of all the evidence must show that it was based on the totality of the evidence. Facts established by rebuttal evidence must be evaluated along with the facts on which the conclusion was reached, not against the conclusion itself. See MPEP § 2142 citing *In re Eli Lilly & Co.*, 902 F.2d 943, 14 USPQ2d 1741 (Fed. Cir. 1990).

In this case, Appellants have pointed out that: (1) Watkins does not provide a specific example wherein an HDAC inhibitor is used to treat osteoarthritis and does not provide an enabling disclosure for such a method of treatment; (2) the references referred to by Watkins in support of the statement that the method of treatment of osteoarthritis using HDACi's was well known do not even mention these conditions and thus do not support the assertion made by Watkins; (3) none of the other references of record indicate that osteoarthritis is "known to be

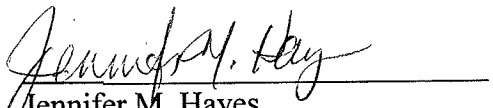
mediated by HDAC" or "known to be treated by HDAC inhibitors"; and (4) the Examiner has not pointed to any other references in support of his position that Watkins is enabling for the treatment of osteoarthritis.

On the other hand, the Examiner has made statements regarding the disclosure of Watkins which are contradicted by objective evidence of the knowledge and skill available in the art. Accordingly, the evidence presented by Appellants is more convincing than the mere statements of the Examiner. Thus, patentability of the present claims is supported by a preponderance of the evidence when the totality of the record is properly taken into consideration.

Accordingly, Appellants respectfully submit that the anticipation rejection should be reversed.

The USPTO is directed and authorized to charge the statutory fee (37 C.F.R. §41.37(a) and 1.17(c)) and all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



Jennifer M. Hayes
Registration No. 40,641

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860
WASHINGTON DC SUGHRUE/265550
65565
CUSTOMER NUMBER

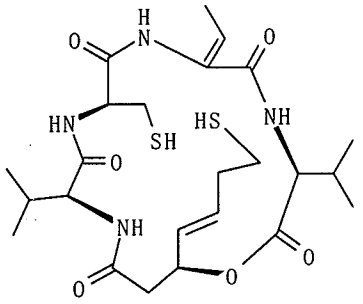
Date: October 3, 2008

CLAIMS APPENDIX

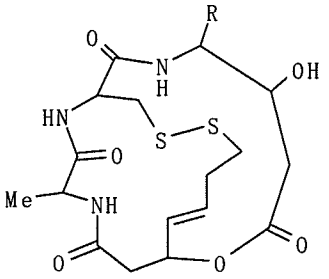
CLAIMS 18-21 ON APPEAL:

18. A method for treating osteoarthritis caused by articular cartilage extracellular matrix degradation, which comprises administering a therapeutically effective amount of a histone deacetylase-inhibiting compound to a patient in need thereof.

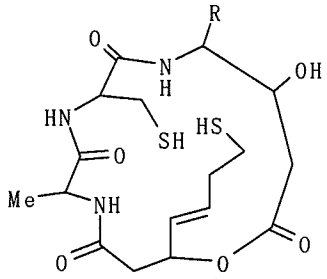
19. The method according to claim 18, wherein the histone deacetylase-inhibiting compound is selected from FK228, MS-27-275, Trichostatin A, NVP-LAQ824, SAHA, Apicidin, Phenylbutyrate, Valproic acid, Pivaloyloxymethyl butyrate, CI-994, Depudecin, Trapoxin, a CHAP, butyric acid and a depsipeptide compound represented by the following formula (I), a depsipeptide compound represented by the following general formula (II), and a depsipeptide compound represented by the following general formula (IIa):



(I)



(II)



(IIa)

wherein R represents an isopropyl group, a sec-butyl group, or an isobutyl group.

20. The method according to claim 19, wherein the histone deacetylase-inhibiting compound is selected from FK228, the depsipeptide compound represented by formula (I), the depsipeptide compound represented by formula (II), the depsipeptide compound represented by

formula (IIa), MS-27-275, Trichostatin A, NVP-LAQ824, SAHA, Apicidin, Phenylbutyrate, and Valproic acid.

21. The method according to claim 18, wherein the histone deacetylase-inhibiting compound is a compound whose histone deacetylase inhibitory activity (IC_{50} value) is a concentration of 100 μM or less measured by a histone deacetylase inhibition assay comprising:

(a) pre-incubating the histone deacetylase-inhibiting compound with [^{3}H] acetyl-histones in a solution containing PTT for 1 hour at room temperature,

(b) adding histone deacetylase to the solution of step (a) and incubating at room temperature for 2 hours, and

(c) measuring the released [^{3}H].

EVIDENCE APPENDIX:

Pursuant to 37 C.F.R. § 41.37(c)(1)(ix), submitted herewith are copies of any evidence submitted pursuant to 37 C.F.R. §§ 1.130, 1.131, or 1.132 or any other evidence entered by the Examiner and relied upon by Appellant in the appeal.

These documents have been submitted:

(1) Dangond et al., "Differential display cloning of HDAC3 cDNA from PHA-activated immune cells", 1998, Biochem. Biophys. Res. Commun., Vol 242, No.3, pp. 648-652 submitted as Attachment 1 with the Amendment filed January 31, 2008.

(2) Takahashi et al., "Selective Inhibition of IL-2 Gene Expression by Trichostatin A, a Potent Inhibitor of Mammalian Histone Deacetylase" 1996, J. Antibiot. (Tokyo), Vol. 49, No.5, pp. 453-457) submitted as Attachment 2 with the Amendment filed January 31, 2008.

(3) Partial English translation of New Integrated Medical Lectures, Classification of Autoimmune Diseases, submitted as Reference Document 3 with the Amendment filed November 27, 2006.

(4) Partial English translation of Orthopedic Surgery, No. 42, submitted as Reference Document 4 with the Amendment filed November 27, 2006.

RELATED PROCEEDINGS APPENDIX

Submitted herewith are copies of decisions rendered by a court or the Board in any proceeding identified about in Section II pursuant to 37 C.F.R. § 41.37(c)(1)(ii).

None

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 242, 648–652 (1998)
ARTICLE NO. RC978033

Differential Display Cloning of a Novel Human Histone Deacetylase (HDAC3) cDNA from PHA-Activated Immune Cells[1]

Fernando Dangond,*,[2] David A. Hafler,* Jeffrey K. Tong,† Jeffrey Randall,‡ Ryoji Kojima,‡ Nalân Utku,‡ and Steven R. Gullans‡

*Department of Neurology and ‡Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115; and †Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

Received December 23, 1997

The nucleosomal histones can be modified through reversible acetylation by histone acetyltransferases (HATs) and deacetylases (HDACs). HATs induce nucleosomal relaxation and allow DNA-binding by transcriptional activators. HDACs form corepressor complexes which negatively regulate cell growth. However, the HDAC inhibitors butyrate and Trichostatin A block T cell proliferation, suggesting that not all effects of HDACs lead to repression. Using mRNA differential display and 5'RACE we isolated human HDAC3, a novel gene that is upregulated in PHA-activated T cell clones. HDAC3 is homologous to other human HDACs and yeast *RPD3*. In peripheral blood mononuclear cells (PBMCs), activation by PHA, PMA and α-CD3 increased HDAC mRNA but no effect was seen with IFN-γ, LPS, or IL-4. In contrast, GMCSF downregulated PBMC levels of HDAC3 mRNA. All HDACs were found to be ubiquitously expressed in immune and non-immune tissues. In human myeloid leukemia THP-1 cells, HDAC3 transfection resulted in increased size, aberrant nuclear morphology and cell cycle G2/M cell accumulation. Functional activity of the expressed HDAC3 protein was confirmed in α-HDAC3 antibody immunoprecipitates by a histone deacetylase assay. Our study suggests the participation of HDACs in cell cycle progression and activation. © 1998 Academic Press

The core histones are structural components of nucleosomes that play key regulatory functions, as their N-terminal domains are post-translationally modifiable through reversible acetylation of their lysine residues [1, 2]. The dynamic equilibrium of lysine acetylation depends on two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Hyperacetylated histones decondense chromatin and make the DNA accessible to transcriptional activation complexes [3, 4]. Deacetylation of histones counteracts this effect by limiting accessibility of activation factors [5, 6] and allowing the binding of known transcriptional repressor complexes to DNA, of which HDACs themselves are part [7, 8].

Recent work identified two human HDACs [9, 10] that form molecular repressor complexes by associating with molecules such as Mad [11], mSin3 [12, 13], YY1 [10] and the nuclear receptor corepressor N-CoR [8]. In addition, studies with HDAC inhibitors such as butyrate and trichostatin A (TSA) substantiated the central role of histone acetylation in gene transcription and differentiation [14–17]. Furthermore, these inhibitors induce specific cell cycle arrest at G1 and G2 phases [17], highlighting the complex regulatory role played by HDACs.

In the present study we used mRNA differential display of PHA-stimulated T cell clones and 5'RACE to isolate HDAC3, a novel HDAC gene whose expression in immune cells appears to be regulated during activation. Moreover, HDAC3 overexpression disturbs cell cycle progression in human cells.

MATERIALS AND METHODS

Isolation of T cell clones. Myelin basic protein (MBP) reactive T cell clones isolated by limiting dilution have been extensively characterized [18–20]. $CD4^+$ T cell clones Ob1A12.8, Ob3D1.7, HyG11.8 and A192 are DR2 restricted and recognize epitope MBP_{85-99}; the HTLV-I-reactive $CD8^+$ T cell clone KS.2E11.7 is A2 restricted and directed against epitope Tax_{11-19} [21].

Studies with activation and growth factors. PBMCs were isolated from healthy donors using a Ficoll-paque (Pharmacia) gradient cen-

[1] The sequences in this paper have been deposited in GenBank (Accession No. U66914).

[2] Corresponding author. Department of Neurology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Fax: (617) 525-5711. E-mail: dangond@cnd.bwh.harvard.edu.

0006-291X/98 $25.00

trifugation. Cells were cultured in RPMI supplemented with 10% human AB serum, 10% IL-2-containing T-Stim (Collaborative Biomedical Products), 2 mM L-glutamine, 10 mM Hepes, and 100 U/100 μg per ml penicillin/streptomycin in Petri dishes at 1×10^6 cells/ml. To assess the response of HDACs to growth and differentiation inducers, PBMCs were stimulated for 48 hours with rIL-4 (Boehringer Mannheim) at 10 ng/ml, rIFN-γ (Gibco-BRL) at 10 ng/ml, or rGMCSF (Promega) at 5 ng/ml. To assess the response to polyclonal activators, we used PMA (Sigma) at 10 ng/ml, PHA (Murex Diagnostics, Dartford, UK) (1 μg/ml), or α-CD3 (OKT3) Abs (Ortho Biotech, Inc., Raritan, N.J.) at 1 ng/ml. Petri dishes had been coated with α-CD3 (1 ng/ml) the night prior and washed twice with PBS, before adding the cells. LPS (Sigma) was used at 10 μg/ml as a control for rIFN-γ-induced activation. Cells were recovered after 48 hours by mechanical dislodging and viability (>95%) was assessed by trypan blue exclusion. Human myeloid leukemia THP-1 cells were grown in RPMI media supplemented with 10% FCS, 10 mM Hepes, 2mM L-glutamine, and 100 U/100 μg per ml penicillin/streptomycin.

RNA isolation and Northern analysis. Total RNA was isolated from T cell clones and PBMCs using the RNAzol B method (Tel-Test, Inc). Northern blots with 20 μg of total RNA per lane were prepared and probed as described [22]. The blots were washed twice at room temperature (2× SSC, 0.1% SDS, 20 min), once at 60°C (0.2× SSC, 0.1% SDS, 20 min), and autoradiographed. An L3 ribosomal cDNA probe was used to verify equivalent loading. Blots containing poly(A)$^+$ RNA (2 μg) were purchased (Clontech) and probed for tissue distribution studies. Hybridizations with either the 343 bp cDNA fragment or the R98879 EST insert yielded identical results. The HDAC1 cDNA probe was kindly provided to us by Stuart Schreiber and Christian Hassig (Harvard University). From Genome Systems (St. Louis, MO) we obtained the cDNA for HDAC2 (EST F06693) to use as a probe. We used a human p53 cDNA probe obtained by PCR as a positive control for rIFN-γ-induced activation [23].

Isolation of HDAC3. mRNA differential display was performed as described [22] using the primers 5′-GATGCCACCATGG-3′ and 5′-AATAAACGCCATT-3′. The differentially expressed cDNA fragment was excised and eluted from the gel, reamplified, cloned into a TA vector (Invitrogen) and sequenced. An identical cDNA (EST R98879) was identified by a dbEST database search and obtained from Genome Systems. The missing 5′ end of the cDNA was obtained by performing 5′RACE (Gibco, BRL) and the full-length cDNA was constructed using an overlapping PstI restriction site and ligation into the pCDNA3.1Zeo vector (Invitrogen). Sequencing was performed at the Howard Hughes Biopolymers Research Facility at Harvard Medical School.

HDAC3 transfection. The pCDNA3.1Zeo and the pCDNA3.1Zeo-HDAC3 constructs were transfected into THP-1 cells by electroporation with a Biorad electroporator (300 V and 1,000 microFarads). Stable transfectants were selected with Zeocin-containing media. Changes in morphology were observed with a Nikon phase contrast microscope.

CC analysis and flow cytometry. Cells were fixed in 70% ethanol and Propidium Iodide (PI) was added following the protocol described by Nicoletti [24], with minor modifications. Analysis of cellular DNA content was performed in a Beckton Dickinsnn FACS sorter.

Histone deacetylase activity assay. For the HDAC activity assay, THP-1 cells were grown and collected 2 days following change of culture media, and PBMC's were collected 2 days after PHA stimulation. Cells were spun down, washed with ice cold PBS, spun down again at 1000 × g for 5 minutes. The cells were then lysed in 400 μl lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.2 nM PMSF) for 30 min. at 4 °C with gentle inversion. Lysates were centrifuged at 14,000 × g for 2 min. to pellet insolubles. The supernatants were collected and aliquoted to 2 tubes each. Rabbit α-HDAC3 antisera (30 μl, kindly provided by Christian Hassig and Stuart Schreiber, Harvard University) was added to each tube followed by 1 hr incubation on ice. Protein A agarose (40 μl) (Gibco, BRL) was added to each tube and tubes were rotated at 4 °C for 3 hr. Beads were then washed 3× with 500 μl lysis buffer. Beads were then resuspended in 200 μl histone deacetylase buffer (50 mM Tris pH 7, 150 mM NaCl, 10% glycerol, 1 mM EDTA). This suspension was then split to 2 tubes of 100 μl each. Trichostatin A (Wako Pure Chemicals) was added to 200 nM final concentration, followed by incubation on ice for 15 minutes. ^{3}H acetylated histone substrate was then added to all tubes and reactions proceeded at 37 °C with vigorous shaking for 3 hr. Reactions were terminated by the addition of 50 μl 0.1 M HCL, 0.16 M AcOH followed by 600 μl ethyl acetate. Tubes were vortexed and spun in a microcentrifuge to separate aqueous layers. The organic phase (400 μl) was added to 3 ml scintillation fluid (DuPont EcoScint) and counted in a scintillation counter (Beckman).

RESULTS AND DISCUSSION

Using mRNA differential display we identified a cDNA present in four chronically PHA-activated T cell clones (Ob1A12.8, Hy3D1.7, HyG11, and KS.2E11.7) but not present in a freshly isolated T cell clone (A192) that had undergone fewer rounds of PHA stimulation. This 343 bp cDNA fragment was cloned, sequenced, and compared to the NCBI-GenBank database using BLASTX. The deduced amino acid sequence was found to have significant homology to the amino terminus of the two known human HDAC cDNAs and the mouse HDAC1 and yeast RPD3 cDNAs suggesting it encoded a new family member. Comparison to the Expressed Sequence Tag (EST) database revealed several identical overlapping human cDNAs. From Genome Systems Inc. (St. Louis, MO) we obtained a 1.8 kb human EST cDNA clone (#R98879) whose 5′ end overlapped with our clone. We performed 5′RACE on mRNA from PHA-stimulated PBMCs and obtained the remaining sequence and an additional 75 nt of the 5′ UTR such that the full-length HDAC3 cDNA is 1984 bp long with an ORF of 1284 nt.

The deduced 428 amino acid sequence of HDAC3 (Fig 1A) predicts a molecular mass of 48,750 Da. It is 50-53% identical to the human HDAC1 [9], HDAC2 [10], and the RPD3 gene from yeast [25]. The homology tree arialysis of the protein sequences (Fig. 1B) revealed that human HDAC3 is more similar to yeast RPD3 than other human HDACs or the other murine, frog, and *Drosophila* homologs. The sequence homology among HDACs spans most of the molecule but the carboxyl-terminus is non-conserved, highly charged and enriched in glutamate and aspartate residues. Hydrophobicity analysis using the Kyte-Doolittle algorithm confirmed the structural similarity among the human and yeast HDACs (Fig. 1C).

To identify tissues that express HDACs, we probed northern blots of immune and non-immune tissues. All HDACs were ubiquitously expressed (Fig. 2) but the levels in non-immune tissues was less uniform, with the highest relative levels of HDAC3 in heart. Thus, the HDACs have tissue-specific expression profiles, but

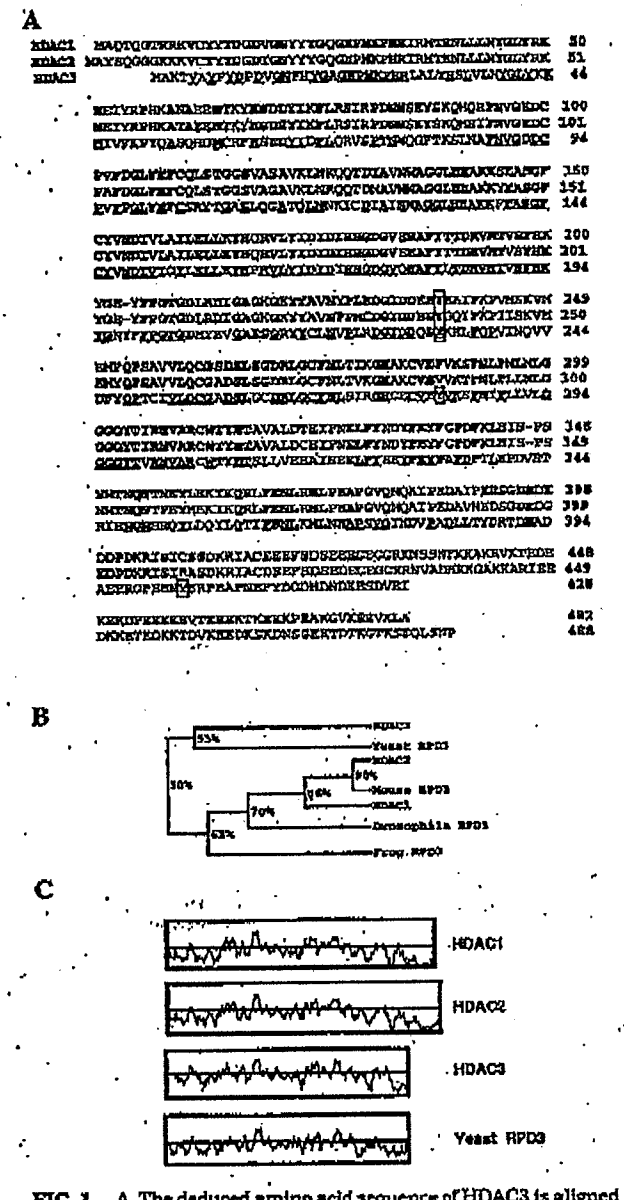


FIG. 1. A. The deduced amino acid sequence of HDAC3 is aligned with other human HDAC sequences. Identical residues are indicated by bold letters. Underlined are those residues that are also conserved with RPD3 proteins of yeast and frog. Boxed residues are putative tyrosine kinase phosphorylation sites, one of which (Y232) is conserved among all members of the family in eukaryotes. B. Phylogenetic tree of the HDAC/RPD3 family of proteins based on the unweighted pair group method with arithmetic mean algorithm. Sequences are from: frog RPD3 (X78454), human HDAC1 (U66914), human HDAC2 (U66914), Drosophila (Y09258) and yeast RPD3 (Z71606). The lengths of the horizontal lines are proportional to the estimated genetic distances. The numbers indicate the average percentage identity among different proteins beyond the branch point. C. Hydrophobicity plot reveals structural homology among human and yeast HDACs.

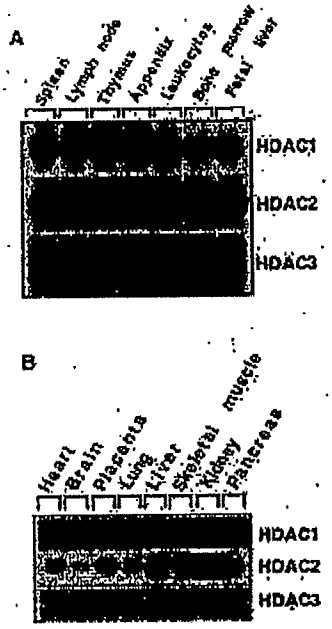


FIG. 2. A. Poly(A)$^+$ RNA northern blots of multiple tissues and HDAC expression. A. There is ubiquitous HDAC family expression in the immune system. B. Analysis of multiple organ tissues reveals less uniform HDAC expression.

the precise functional significance of this finding remains unclear.

To assess whether HDAC mRNA levels increased upon immune cell activation, freshly-isolated PBMCs were exposed to a variety of stimuli for 48 hours. Expression of all three HDACs was increased by PMA and PHA (Fig. 3A), which induce proliferation and increase expression of p53 [26-28], and by α-CD3. As shown in Fig. 3B, IL-4 and LPS failed to elicit large changes in expression of HDACs and had no effect on p53 expression. IFN-γ did not increase HDAC expression though p53 mRNA level was enhanced. These observations extend the recent demonstration that murine HDAC1 expression is increased by IL-2 in the B6.1 cytolytic mouse cell line and this correlates with enhanced proliferation [29]. Surprisingly, GMCSF caused a large reduction in HDAC3 expression, suggesting that suppression of HDAC activity may be involved in GMCSF-induced differentiation [30].

To evaluate histone deacetylase activity of HDAC3, α-HDAC3 antibodies were used to immunoprecipitate HDAC3 from actively growing human THP-1 and PHA-stimulated immune cells. As shown in Figure 4, immunoprecipitated HDAC3 protein exhibited TSA inhibitable histone deacetylase activity. Furthermore, overexpression of HDAC3 in transfected THP-1 cells led to an accumulation in G2/M phase (Fig. 5), with a significant

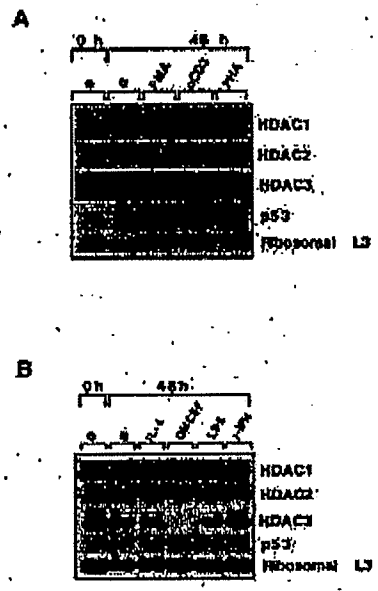


FIG. 3. Northern analysis of peripheral blood mononuclear cells (PBMCs) shows that HDAC mRNA is modified by polyclonal activators and GMCSF. A. PBMCs were cultured with or without PMA, PHA, or α-CD3. B. HDAC3 expression is downregulated by GMCSF at 48 hours but not significantly altered by γ-IFN, IL-4 or LPS. Not shown, the downregulation by GMCSF was seen at earlier time points (6 and 24 hours) for HADC1 and 3 but not for HDAC2. A p53 cDNA probe was used as a control in (A) and (B). Regulation of HDACs by polyclonal activators and GMCSF was confirmed by analyzing PBMCs mRNA from three healthy donors in three separate northern blot experiments.

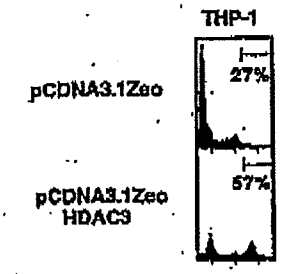


FIG. 5. FACS analysis of DNA content reveals cell cycle abnormalities induced by HDAC3 overexpression. Stably-transfected THP-1 cell lines exhibit decreased accumulation in the G0/G1 phase, no increase in the relative percentage of cells in the early S phase, and a distinct accumulation of cells in the G2/M phase (three independent experiments yielded consistent findings). Relative percentages of cells in G2/M are shown. Necrotic cells were excluded from the analysis. Ten thousand cells were counted for each sample.

increase in cell size and aberrant nuclear morphology (not shown) suggesting an inability of these cells to progress coordinately and undisturbed through the cell cycle and a poor integration of size control mechanisms. Our results are consistent with the G2/M cell cycle abnormalities observed when transfecting mouse HDAC1 into Swiss 3T3 cells [29]. Of note, both in plant [31] and yeast [32], modification of histones to prevent acetylation results in G2/M abnormalities, suggesting a role for HDACs in modulation of the mitotic stage.

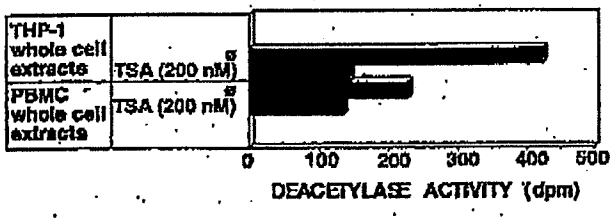


FIG. 4. Histone deacetylase activity assay. Human THP-1 myeloid leukemia cells and PHA-stimulated PBMC's were collected after 48 hrs in culture media. Whole cell extracts were analyzed in duplicate for ability to deacetylate [^{3}H]acetylated histone substrate, and [^{3}H]acetic acid release was expressed as the average in dpm.

Identifying the transcriptional "switches" that define the phenotype of activated immune cells will likely provide a better understanding of the molecular mechanisms underlying immunity. Histone acetylation and deacetylation are important determinants of gene transcription and cell differentiation. The strong evolutionary conservation of HDACs suggests they play a fundamental role in multiple and complex cellular pathways of immune system regulation, including cell activation, growth and differentiation, and thus represent potential molecular targets for the treatment of cancer and autoimmunity. Detailed studies to define the specific immune cell types involved in HDAC upregulation upon activation and the mechanisms responsible for HDAC-induced cell cycle abnormalities are now under way.

ACKNOWLEDGMENTS

The authors thank Drs. Bento Santos and Alejandro Chevaile for helpful suggestions and technical help, and Dr. Lara Ausubel for providing an antigen-reactive T cell clone derived from a normal individual. This work was supported by a Howard Hughes Physician Scientist Post-doctoral Fellowship (F.D.), a NSF Pre-doctoral Fellowship and the Harvard-Markey Biomedical Scientist Program (J.K.T.), and National Institutes of Health Grants DK36031 (S.R.G.) and NS24247 (D.A.H.).

REFERENCES

1. Johnson, L. M., Kayne, P. S., Kahn, E. S., and Grunstein, M. (1990) *Proc. Natl. Acad. Sci. USA* 87, 6286–6290.
2. Megee, P. C., Morgan, B. A., Mittman, B. A., and M. M., S. (1990) *Science* 247, 841–845.
3. Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1988) *EMBO J.* 7, 1395–1402.
4. Lee, D. Y., J. J., H., Pruss, D., and Wolffe, A. P. (1993) *Cell* 72, 73–84.

5. Roth, S. Y., and Allis, C. D. (1996) *Cell* 84, 843–851.
6. Wolffe, A. P., and Pruss, D. (1996) *Cell* 84, 817–819.
7. Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D., and Broach, J. R. (1993) *Genes Dev.* 7, 592–604.
8. Heinzel, T., Lavinsky, R. M., Mullen, T.-M., Söderström, M., Laherty, C. D., Torchia, J., Yang, W.-M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* 387, 43–48.
9. Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996) *Science* 272, 408–411.
10. Yang, W.-M., Inouye, C., Zeng, Y. Y., Bearss, D., and Seto, E. (1996) *Proc. Natl. Acad. Sci. USA* 93, 12845–12850.
11. Laherty, C. D., Yang, W.-M., Sun, J.-M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997) *Cell* 89, 349–356.
12. Hassig, C. A., Fleischer, T. C., Billin, A. N., Schreiber, S. L., and Ayer, D. E. (1997) *Cell* 89, 341–347.
13. Zhang, Y., Iratni, R., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (1997) *Cell* 89, 357–364.
14. Van Lint, C., Emiliani, S., Ott, M., and Verdin, E. (1996) *EMBO J.* 15, 1112–1120.
15. Hoshikawa, Y., Kwon, H. J., Yoshida, M., Horinouchi, S., and Beppu, T. (1994) *Exp. Cell Res.* 214, 189–197.
16. Candido, E. P., Reeves, R., and Davie, J. R. (1978) *Cell* 14, 105–113.
17. Yoshida, M., Horinouchi, S., and Beppu, T. (1995) *Bioessays* 17, 423–430.
18. Ota, K., Matsui, M., Milford, E. L., Mackin, G. A., Weiner, H. L., and Hafler, D. A. (1990) *Nature* 346, 183–187.
19. Wucherpfennig, K. W., Sette, A., Southwood, S., Oseroff, C., Matsui, M., Strominger, J. L., and Hafler, D. A. (1994) *J. Exp. Med.* 179, 279–290.
20. Wucherpfennig, K. W., and Strominger, J. L. (1995) *Cell* 80, 695–705.
21. Höllsberg, P., Weber, W. E. J., Dangond, F., Batra, V., Sette, A., and Hafler, D. A. (1995) *Proc. Natl. Acad. Sci. USA* 92, 4036–4040.
22. Kojima, R., Randell, J., Brenner, B. M., and Cullans, S. R. (1996) *J. Biol. Chem.* 271, 12327–12332.
23. Kano, A., Watanabe, Y., Takeda, N., Aizawa, S., and Akaike, T. (1997) *J. Biochem. (Tokyo)* 121, 677–683.
24. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) *J. Immunol. Methods* 139, 271–279.
25. Vidal, M. S., and Gaber, R. F. (1991) *Mol Cell Biol* 11, 6317–6327.
26. Mateo, M. S., Sanchez-Beato, M., Martinez, J. C., Orfao, A., Orradre, J. L., and Piris, M. A. (1995) *J. Clin. Pathol.* 48, 151–159.
27. Reed, J. C., Alpers, J. D., Nowell, P. C., Hoover, R. G. (1986) *Proc. Natl. Acad. Sci. USA* 83, 3982–3986.
28. McCubrey, J. A., Steelman, L. S., and McKearn, J. P. (1991) *Oncogene Res.* 6, 1–12.
29. Bartl, S., Taplick, J., Lagger, G., Khier, H., Kuchler, K., and Seiser, C. (1997) *Mol. Cell Biol.* 17, 5033–5043.
30. Socinski, M. A., Cannistra, S. A., Sullivan, R., Elias, A., Antman, K., Schnipper, L., and Griffin, J. D. (1988) *Blood* 72, 691–697.
31. Belyaev, N. D., Houben, A., Baranczewski, P., and Schubert, I. (1997) *Chromosoma* 106, 193–197.
32. Kayne, P. S., Kim, U.-J., Han, M., Mullen, J. R., Yoshizaki, F., and Grunstein, M. (1988) *Cell* 55, 27–39.

Selective Inhibition of IL-2 Gene Expression by Trichostatin A, a Potent Inhibitor of Mammalian Histone Deacetylase

ISAMI TAKAHASHI*, HIROMASA MIYAJI, TETSUO YOSHIDA, SOICHIRO SATO† and TAMIO MIZUKAMI

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan.
†Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
1188 Shimotogari, Nagaizumi-cho, Shizuoka 411, Japan.

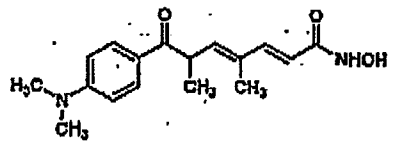
(Received for publication October 6, 1995)

During screening for inhibitors of T cell activation, we have found that trichostatin A (TSA), known as a potent inhibitor of histone deacetylase, showed selective inhibitory activity against IL-2 gene expression. From luciferase reporter experiments on human leukemic Jurkat T cells, TSA was found to inhibit the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter with a 50% inhibitory concentration value of 0.073 μM. On the other hand, TSA, at the same concentration, enhanced the expression of the luciferase reporter gene directed by the c-*fos* enhancer and promoter. The result of RT-PCR experiments also indicates that TSA has selective inhibitory activity against IL-2 gene expression in Jurkat cells. These results suggest that the change in chromatin structure caused by the hyperacetylation of histone might affect the regulation of IL-2 and c-*fos* gene expression.

Activation of T lymphocytes is a complex process requiring recognition of the antigen by the T cell antigen receptor as well as concomitant costimulatory signals from antigen presenting cells. These events induce the activation of second messenger pathways such as tyrosine kinases and protein kinase C and lead to an increase in intracellular Ca^{2+}. These signals finally give rise to a sequential activation of sets of genes that in turn initiate proliferation, differentiation and immunological functions[1]. Although detailed mechanisms for these events are still unclear, recent studies concerning the molecular actions of cyclosporin A(CsA)[1] and FK506 have provided important new information on the biochemical process which regulates these events. Of special significance has been the realization that these molecules act as "molecular adaptors" which serve to mediate the interaction between their respective intracellular drug-binding proteins (or "immunophilins") and their individual target molecules. Vigorous research on the mode of action of CsA and FK506 has shown that the heterodimeric, Ca^{2+}/calmodulin-regulated phosphatase calcineulin is a major common target of the CsA-cyclophilin A and FK506-FK506 binding protein 12 (FKBP12) drug-immunophilin complex *in vitro* and that drug-immunophilin complex blocks the dephosphorylation by calcineulin of the nuclear factor of activated T cells (NFATc) in cytoplasm, a step that is required for its translocation to the nucleus. The drug-immunophilin complexes with calcineulin and inhibition of its phosphatase activity provide a molecular basis for the inhibitory effect of CsA or FK506 on expression of gene encoding IL-2 and other cytokines[2]. Although several signaling inhibitors have been reported for the effect of the gene expression, as of now no selective inhibitor of IL-2 gene expression is known other than CsA and FK506 (manuscript in preparation).

Trichostatin A (TSA), which had been originally found from its antifungal activity[3], was shown by YOSHIDA *et al.* to induce differentiation of Friend leukemia cell and to inhibit the cell cycle of normal rat fibroblasts in the G1 and G2 phases at low concentration[4,5], and shown by SUGITA *et al.* to induce morphological reversion of *sis*-transformed NIH3T3 cells[6]. YOSHIDA *et al.* also found that the target molecule of TSA was histone deacetylase that is potently and specifically inhibited by

Fig. 1. Structure of trichostatin A.



Trichostatin A

TSA[7]. Since histone proteins have an essential supporting role in the transcriptional machinery for regulating gene expression, the relationship between core histone hyperacetylation caused by the inhibition of the histone deacetylase and several gene expressions is receiving increasing attention[8]. It has been reported that TSA which induces histone hyperacetylation affects the gene expression of gelsolin[9], histone H1[0 10], cytokeratin A (*endo* A)[11] and early gene products (c-*jun*, c-*fos* and c-*myc*)[12].

We report here that TSA shows inhibitory activity against the IL-2 gene expression and enhancing activity against the c-*fos* gene expression, and that TSA has immunosuppressive activity in a mouse experimental model.

Materials and Methods

Isolation of Trichostatin A (TSA)

A reporter gene assay, which is luciferase assay described below, has been used to screen for isolation of TSA. The TSA producing organism was isolated from soil collected in Japan and was taxonomically classified as *Streptomyces* sp. GT15. TSA was accumulated in both the mycelium and culture filtrate. After adjustment to pH 4.0 with 6N HCl, the culture filtrate (30 liter) was applied to a column of Diaion HP-20 (2 liter) (Mitsubishi Chemical Industries Limited). The column was washed with deionized water and 30% methanol (MeOH) and then eluted with 100% MeOH. After concentration, the eluate was extracted with normal-butyl alcohol (*n*-BuOH). The extract was concentrated and the residue was subjected to silica gel (Merck Art. No. 7734) column chromatography using the stepwise method of chloroform ($CHCl_3$)-MeOH as eluting solvents. The active fractions were combined and evaporated to dryness. The residue was rechromatographed on silica gel (Merck Lichroprep Si 60) with $CHCl_3$-MeOH, and the active fractions were further purified with HPLC using a packed column (YMC-ODS SH-365-5, 65% MeOH) to yield 63 mg of TSA.

Cell Lines and Culture

Jurkat cells were maintained in complete RPMI1640 (Gibco) supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml) in a 5% CO_2 air atmosphere. Jurkat/pIL2luc2 #15 and Jurkat/pfosluc22 #39 were periodically cycled in the above media with 0.3 mg/ml hygromycin B.

Plasmid Construction and Luciferase Assays

Plasmid construction, transfection and selection of hygromycin-resistant Jurkat clones were described by MIYAJI *et al.* (manuscript in preparation). Jurkat/pIL2luc2 #15 cells were stimulated in 200 μl fresh culture media with 12-*O*-tetradecanoylphobol-13-acetate (TPA) (5 ng/ml) and phytohemagglutinin (PHA) (1 μg/ml) in a tube and incubated for 6 hours at 37°C. Jurkat/pfosluc22 #39 cells were stimulated in 200 μl fresh culture media with TPA (5 ng/ml) and PHA (1 μg/ml) in a tube and incubated for 3 hours at 37°C. TSA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and used at the indicated concentrations. After incubation, luciferase activities were measured using a luminometer (EG&G Berthold Autolumat LB953).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA samples were reverse transcribed with Superscript RT (Bethesda Research Laboratories, Rockville, MD) and IL-2 or c-*fos* specific primers (STRATAGENE) were used to amplify selected IL-2 or c-*fos* with AmpliTaq DNA polymerase (Cetus corpration).

Mixed Lymphocyte Reaction (MLR)

Mice were obtained from Nihon SLC. MLR were set up as previously described by WEBB *et al.*[13]. 1.5×10^5 Lymph node responder cells from B10.BR mice were cultured with 5×10^5 mitomycin C-treated spleen stimulator cells from AKR mice. The culture was pulsed with 1 μCi per well of [^{3}H]thymidine approximately 18 hours before harvesting onto fiber filter-mats, and then counted using a scintillation counter.

Antiproliferation Activity

For determination of the antiproliferative activity of TSA, Jurkat/pIL2luc2 #15 was cultured in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated into 96-well microtiter plate (10^4 cells/well) in the presence of various concentrations of TSA and incubated for 72 hours. Culture was pulsed with 1 μCi per well of [^{3}H]thymidine 18 hours before harvesting onto filter-mats, and counted in a scintillation counter. The results were expressed as an IC_{50}, the drug concentration required for 50% inhibition of cell growth. HeLa S3 cells were cultured in DULBECCO's modified EAGLE's medium containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). HeLa S3 cells were preincubated for 24 hours at 37°C in 96-well dishes and then treated with different dilutions of TSA for 3 days. Thereafter, the concentration of TSA required for 50% inhibition of cell growth was determined by Gimsa staining[14].

Delayed-type Hypersensitivity (DTH)[15]

To induce DTH to trinitrophenyl (TNP), 0.1 ml of 10 mM trinitrobenzene sulfonic acid (TNBS) solution (pH 7.4) was subcutaneously injected into two separate sites on the dorsal flanks of the Balb/c mice. The compound dissolved in 10% DMSO was injected intraperitoneal (i.p.) once a day through day 0 to day 4. Ten percent DMSO was used as the control. Challenge was performed

5 days later by injecting 0.05 ml of 10 mM TNBS solution (pH 7.4) into the right footpad. Twenty-four hours after the footpad challenge, DTH reactivity was assessed by measuring the swelling of the footpad. The magnitude of the DTH was expressed as the increment of the thickness of the challenged right footpad as compared with the untreated left footpad. Each group consisted of five mice. TSA was dissolved in DMSO and administered by i.p. injection for 5 consecutive days, beginning on the day of sensitization.

Results and Discussion

In the course of screening for inhibitors of the T cell signal transduction pathway leading to IL-2 gene expression, we have found that a strain of *Streptomyces* sp. GT15 produced a compound which showed a potent inhibitory activity against the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter. The compound was isolated from the culture broth by the combination of column chromatographies, and identified, based on physico-chemical properties and NMR analysis, as trichostatin A (TSA) previously known as a potent inhibitor of histone deacetylase (Fig. 1).

From the luciferase reporter experiments on Jurkat cells, TSA inhibited the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter with the 50% inhibitory concentration (IC_{50}) value of 0.073 μM. On the other hand, interestingly, TSA enhanced the reporter gene expression directed by the c-*fos* enhancer and promoter at the same concentration as shown in Fig. 2. These potent contrasting activities of TSA were reduced by glycosylation at the position of hydroxamate, trichostatin C[16] (data not shown), indicating that the hydroxamate moiety of TSA is essential for the effects on the reporter gene expression. These results are showing that contrasting activities of TSA on gene expression of IL-2 and c-*fos* were ascribed to the inhibition of histone deacetylase. Because the inhibitory activity of TSA on present gene expression was dependent on the hydoroxamate moiety which is parallel with the effect on histone deacetylase[7] and also there was no common characteristics in the promoter-enhancer regions of the genes irrespective of different sensitivities to TSA.

It has been reported that TSA affects the gene expression of gelsolin[9], histone H1[0 10], cytokeratin A (*endo* A)[11] and early gene products (c-*jun*, c-*fos* and c-*myc*)[12], all of these reported gene expressions, except of c-*jun*, have been significantly enhanced by TSA. Therefore, it is noteworthy that TSA selectively inhibits the IL-2 gene expression.

Before the discovery that TSA is an inhibitor of histone deacetylase, sodium *n*-butyrate was used to inhibit histone deacetylase, although its inhibitory activity is weak[17~20]. We have tried to determine whether sodium *n*-butyrate also shows selective inhibitory activity against the IL-2 gene expression and enhancement activity against the c-*fos* gene expression. Although the concentration of the drug is very high (IC_{50} = 1.0 mM), sodium *n*-butyrate inhibited the IL-2 reporter gene expression and enhanced the c-*fos* reporter gene expression like TSA (Fig. 3). While the observations shown here for sodium *n*-butyrate have not been reported, a rapid alteration of the c-*myc* and c-*jun* gene expression caused by sodium *n*-butylate has been reported[21].

These results indicate that core histone hyperacetylation caused by the inhibition of histone deacetylase may be involved in the alteration of IL-2 and c-*fos* gene expression.

To confirm the selective inhibitory activity against the IL-2 gene expression, the effect of TSA on the endogenous IL-2 mRNA expression on Jurkat cells was investigated using a RT-PCR experiment. As shown in Fig. 4, TSA as well as cyclosporin A (CsA) inhibited induction of endogenous IL-2 mRNA at the concentration of 1 μM, not that of β-actin and G3PDH mRNA as controls. Similarly, induction of endogenous c-*fos* mRNA was

Fig. 2. Effect of trichostatin A on IL-2 and c-*fos* reporter expression.

IL-2 (●) and c-*fos* (■) reporter activities in the presence of various concentrations of TSA.

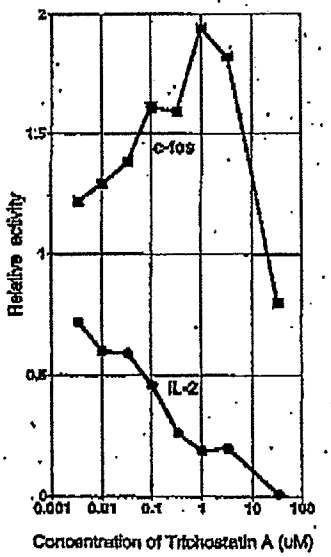


Fig. 3. Effect of sodium *n*-butylate on IL-2 and *c-fos* reporter expression.

IL-2 (●) and *c-fos* (■) reporter activities in the presence of various concentrations of sodium *n*-butyrate.

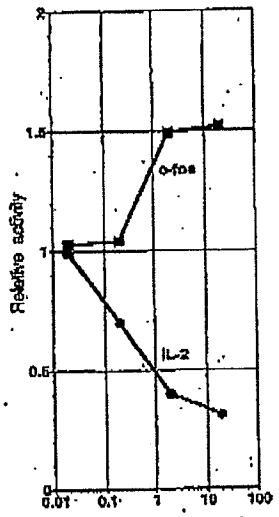
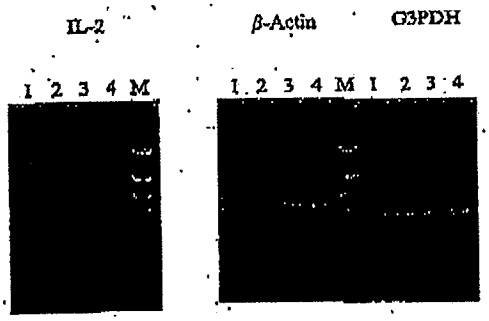


Fig. 4. Effect of trichostatin A on internal IL-2 mRNA expression by RT-PCR.

lane 1: no addition, lane 2: +TPA (5 ng/ml)+PHA (1 μg/ml), lane 3: +TPA (5 ng/ml)+PHA (1 μg/ml)+TSA (1 μM), lane 4: +TPA (5 ng/ml)+PHA (1 μg/ml)+cyclosporin A (1 μM), M: marker.



enhanced by TSA but not by CsA (data not shown). These results indicate the following. First, since the endogenous IL-2 mRNA expression as well as the IL-2 reporter expression were affected by TSA, the activities of TSA against the reporter expression do not appear to be due to the positional effect of a stable transformant of Jurkat cells. Second, the mode of action of TSA is quite different from that of CsA because of its different effect on the *c-fos* gene expression.

IL-2 production on Jurkat cells stimulated by PHA and TPA was also inhibited by TSA using enzyme linked immunosorbent assay (ELISA) (data not shown).

Table 1. Effect of trichostatin A on delayed type hypersensitivity (DTH).

Compound	Dose (mg/kg)			Inhibition(%)
Trichostatin A	100	×5	ip	64.4 (4/5Toxic)
	50	×5	ip	47.1
	25	×5	ip	17.2
	12.5	×5	ip	8.7
Cyclosporin A	30	×5	po	94.5

In order to investigate immunosuppressive activity, TSA was first assessed *in vitro* using the mixed lymphocyte reaction (MLR). As results, TSA showed inhibitory activity against the MLR with an IC_{50} value of 0.032 μM and showed antiproliferative activity against the Jurkat cells with an IC_{50} value of 0.052 μM, while the IC_{50} value of antiproliferative activity against HeLa S3, a nonlymphoidic cell line, was 1.1 μM. Secondly, TSA was assessed *in vivo* using the mouse delayed type hypersensitivity (DTH) experiment. TSA showed 47.1% inhibition by i.p. injection of 50 mg/kg × 5 as shown in Table 1, although the inhibition of TSA against DTH is less effective than that of CsA (30 mg/kg × 5 p.o.: 94.5%).

TSA has a variety of biological activities such as antifungal, induction of cell differentiation, cell cycle arrest, morphological change and effect on gene expression[3~6,9~12]. These biological activities of TSA are considered to be a result of the inhibition of histone deacetylase[7]. However, there is little information on the relation between these biological activities and inhibition of histone deacetylase on gene expression.

The level of histone acetylation is mainly controlled by the acetyltransferase-deacetylase equilibrium[22]. Reversible histone acetylation, which occurs at the ε-amino group of specific internal lysine residues located at the concerned domains of the core histones, is supposed to play an important role in the regulation of the chromatin structure and function specifically in DNA replication and transcription[23]. TSA inhibits histone deacetylase resulting in the hyperacetylation of histone[7]. The hyperacetylation of histone is generally considered to provoke relaxation of the chromatin structure to make various transcriptional factors accessible to DNA. It seems that the transient increase in the site specific or phase dependent histone acetylation may be essential for the early stage of gene expression.

Although it is not clear why the inhibition of histone deacetylase causes the inhibition of IL-2 gene expression and activation of the *c-fos* gene expression, it is of great

interest to know the mechanisms that TSA possesses for contrasting activities against immediate-early (c-*fos*) and early gene (IL-2) expression caused by external stimuli on Jurkat cells. The dramatic change in chromatin structure by the hyperacetylation of histone might affect the DNA binding capability of various transcriptional factors or their regulatory molecules. This might result in the difference between the IL-2 gene expression induced only by specific stimuli from the T cell receptor and the c-*fos* gene expression induced by non-specific stimuli. Future experiments must address how a set of transcriptional factors and/or their regulatory molecules recognize the acetylated form of the chromatin structure and control the IL-2 and/or c-*fos* gene expression to coordinate the complex organization of T cell signal transductional events.

Acknowledgments

The authors deeply thank Ms. Y. Naitoh, Ms. M. Kamiya, Ms. K. Hasegawa and Mr. Y. Nishie for their technical assistance.

References

1) Weiss, A. & D. R. Littman: Signal transduction by lymphocyte antigen receptors. Cell 76(2): 263~274, 1994
2) Schreiber, S. L. & G. R. Crabtree: The mechanism of action of cyclosporin A and FK506. Immunology Today 13(4): 136~142, 1992
3) Tsuji, N.; M. Kobayashi, K. Nagashima, Y. Wakisaka & K. Koizumi: A new antifungal antibiotic, trichostatin. J. Antibiotics 29: 1~6, 1976
4) Yoshida, M.; S. Nimurai & T. Beppu: Effects of trichostatins on differentiation of murine erythroleukemia cells. Cancer Res. 47(14): 3688~91, 1987
5) Yoshida, M. & T. Beppu: Reversible arrest of proliferation of rat 3Y1 fibroblasts in both the G1 and G2 phases by trichostatin A. Exp. Cell Res. 177: 122~131, 1988
6) Sugita, K.; K. Koizumi & H. Yoshida: Morphological reversion of sis-transformed NIH3T3 cells by trichostatin A. Cancer Res. 52: 168~172, 1992
7) Yoshida, M.; M. Kijima, M. Akita & T. Beppu: Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J. Biol. Chem. 265: 17174~17179, 1990
8) Wolffe, A. P.: Transcription: in tune with the histones. Cell 77: 13~16, 1994
9) Hoshikawa, Y.; H. J. Kwon, M. Yoshida, S. Horinouchi & T. Beppu: Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. Exp. Cell Res. 214: 189~197, 1994
10) Girardot, V.; T. Rabilloud, M. Yoshida, T. Beppu, J.-J. Lawrence & S. Khochbin: Relationship between core histone acetylation and histone H10 gene activity. Eur. J. Biochem. 224: 885~892, 1994
11) Miyashita, T.; H. Yamamoto, Y. Nishimune, T. Morita & A. Matsushiro: Activation of the mouse cytokeratin A (*endo* A) gene in teratocarcinoma F9 cells by the histone deacetylase inhibitor Trichostatin A. FEBS Lett. 353: 225~229, 1994
12) Futamura, M.; Y. Monden, T. Okabe, J. Fujita-Toshigaki, S. Yokoyama & S. Nishimura: Trichostatin A inhibits both *ras*-induced neurite outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. Oncogene 10: 1119~1123, 1995
13) Webb, S.; C. Morris & J. Sprent: Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell 63: 1249~1256, 1990
14) Takahashi, I.; S. Nakanishi, E. Kobayashi, H. Nakano, K. Suzuki & T. Tamaoki: Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antiretroviral activity. Biochem. Biophis. Res. Commun. 165: 1201~1212; 1989
15) Tsurufuji, M.; B. Benacerraf & M. Sy: An antigen-specific signal is required for the activation of second-order suppressor T cells in the regulation of delayed-type hypersensitivity to 2,4,6-trinitrobenzene sulfonic acid. J. Exp. Med. 158: 932~945, 1983
16) Tsuji, N. & M. Kobayashi: Trichostatin C, a glucopyranosyl hydroxamate. J. Antibiotics 31: 939~944, 1978
17) Cousens, L. S.; D. Gallwitz & B. M. Alberts: Different accessibilities in chromatin to histone acetylase. J. Biol. Chem. 254: 1716~1723, 1979
18) Candido, E. P. M.; R. Reeves & J. R. Davie: Sodium butyrate inhibits histone deacetylation in cultured cells. Cell 14: 105~113, 1978
19) Sealy, L. & R. Chalkley: The effect of sodium butyrate on histone modification. Cell 14: 115~121, 1978
20) Vidali, G.; L. C. Boffa, E. M. Bradbury & V. G. Allfrey: Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. Proc. Natl. Acad. Sci. U.S.A. 75: 2239~2243, 1978
21) Souleimani, A. & C. Asselin: Regulation of c-*myc* expression by sodium butyrate in the colon carcinoma cell line Caco-2. FEBS Letters 326: 45~50, 1993
22) Csordas, A.: On the biological role of histone acetylation. Biochem. J. 265: 23~38, 1990
23) Allfrey, V. G.; R. Faulkner & A. E. Mirsky: Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc. Natl. Acad. Sci. U.S.A. 51: 786~794, 1964

Partial English translation is as follows.

(page 29)

Table 2-2: Classification of Autoimmune Diseases (Classification by Roitt) Spectrum of Autoimmune Diseases

[Organ-Specific Autoimmune Diseases]
- Hashimoto's disease
- Pernicious anemia
- Autoimmune atrophic gastritis
- Addison's disease
- Goodpasture's syndrome
- Pemphigus
- Pemphigoid
- Sympathetic ephthalmia
- Severe myasthenia
- Autoimmune hemolytic anemia
- Idiopathic thrombocytopenia
- Idiopathic leucopenia
- Primary biliary cirrhosis
- Chronic active hepatitis
- Ulcerative colitis
- Sjögren's syndrome

[Organ-Nonspecific Autoimmune Diseases]
- Systemic erythematosus
- Dermatomyositis
- Scleroderma
- Chronic rheumatic arthritis

NEW INTEGRATED MEDICAL LECTURES

参考資料3
V Reference Document 3

免疫・アレルギー・リウマチ病学

第2版

編集
柏崎禎夫
狩野庄吾

医学書院

自己免疫疾患

自己免疫疾患とはなにか

【自己免疫疾患の定義】

自己免疫疾患(autoimmune disease)は，自己の生体構成成分に対して異常なあるいは過剰な免疫応答が起こり，産生された自己抗体や自己反応性T細胞が関与して発症する疾患である。

【自己免疫疾患の証明】

しかし，自己免疫が原因となって発症しているか否かを厳密に証明することは困難であり，実際には不可能なことが多い。疾患モデル動物について，自己抗体や自己反応性T細胞によって，疾患が他の動物に受身伝達できることを証明できれば，疾患の原因が自己免疫であることはほぼ確実と考えられる。一般には，自己抗体あるいは自己反応性T細胞の証明，標的細胞ないし病変部組織への免疫グロブリンや補体成分の沈着あるいはT細胞や形質細胞の浸潤，血清補体価低値などが自己免疫疾患を示唆する所見と考えられている。

自己抗体や自己反応性T細胞が疾患の原因となっている以外に，疾患による組織損傷の結果，自己抗体や自己反応性T細胞が産生されていることもあり得る。さらに，二次的に産生された自己抗体が，組織傷害を永続化させる働きをしていることもある。

自己免疫疾患の分類

【Roittの分類】

Roittは，自己免疫疾患を臓器特異性自己抗体を持つ臓器特異的自己免疫疾患(橋本病など)と，臓器非特異性自己抗体を持つ臓器非特異的自己免疫疾患(全身性エリテマトーデスなど)を両極とする疾患スペクトラムとして考え，中間に臓器非特異的抗体を持つ臓器限局性疾患(原発性胆汁性肝硬変など)を置いた(表2-2)。

【免疫学的発症機序による分類】

自己抗原の局在によって自己免疫疾患を分類することもできる。細胞表面抗原に対する自己抗体による自己免疫疾患は，臓器特異的ないし臓器限局性自己免疫疾患であることが多く，発症にⅡ型アレルギーまたはⅤ型アレルギーが関与している。汎在性の自己抗原に対する自己抗体によって引き起こされる自己免疫疾患は，一般に臓器非特異的であり，Ⅲ型アレルギーが関与することが多い。

免疫学的発症機序による自己免疫疾患の分類は，Ⅱ型アレルギーによるもの，Ⅲ型アレルギーによるもの，Ⅳ型アレルギーによるもの，Ⅴ型アレルギーによるものに分けられる。

【Ⅱ型アレルギー】

Ⅱ型アレルギーによる自己免疫疾患は，細胞表面の自己抗原に対して抗体が結合した結果，補体活性化またはIgG-Fcレセプターを介した脾臓などの網内系マクロファージによる捕捉が起こり，細胞傷害をもたらす。自己免疫性溶血性貧血や特発性血小板減少性紫斑病が代表例である。糖尿病の一部に，インスリン自己抗体によるインスリンの中和によって起こるものがある。重症筋無力症は，抗アセチルコリンレセプター自己抗体により，アセチルコリンのレセプターへの結合が阻止されることによって発症すると考えられる。

【Ⅲ型アレルギー】

Ⅲ型アレルギーの代表例は，全身性エリテマトーデスである。DNA-抗DNA抗体などの免疫複合体が腎糸球体などの組織に沈着し，補体活性化の結果，好中球やマクロファージが集積し，免疫複合体を貪食してリソソーム酵素や活性酸素を遊離し，組織傷害を引き起こす。

表2-2 自己免疫疾患の分類(Roittの分類) 自己免疫疾患のスペクトラム

臓器特異的自己免疫疾患			臓器非特異的自己免疫疾患
橋本病	類天疱瘡	原発性胆汁性肝硬変	全身性エリテマトーデス
悪性貧血	交感性眼炎	慢性活動性肝炎	皮膚筋炎
自己免疫性萎縮性胃炎	重症筋無力症	潰瘍性大腸炎	強皮症
Addison病	自己免疫性溶血性貧血	Sjögren症候群	慢性関節リウマチ
Goodpasture症候群	特発性血小板減少症		
天疱瘡	特発性白血球減少症		

(page 32)

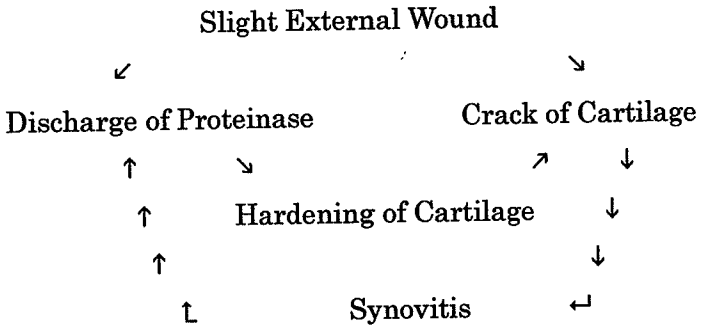


Fig. 4: Onset Mechanism of OA

ISSN 0287-1645
Bessatsu Seikeigeka

参考資料 4
Reference Document 4

別冊整形外科 ORTHOPEDIC SURGERY

No. 42

変形性膝関節症および周辺疾患

「整形外科」編集委員　監修
杏林大学教授　石井良章　編集

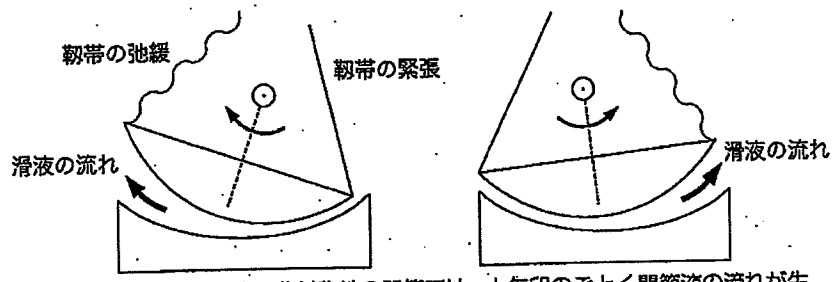


図2. 非対称性の関節. 非対称性の関節では, 太矢印のごとく関節液の流れが生じる. このとき関節が開いている側の靭帯は弛緩し, 閉じている側の靭帯は緊張する.

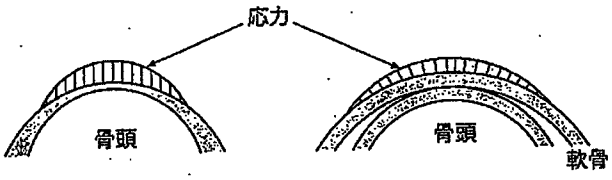


図3. 接触面積と応力分布の関係. 関節軟骨が存在しない場合の計算値と, 軟骨があいだに存在した場合の計算値. 応力は緩和されている.

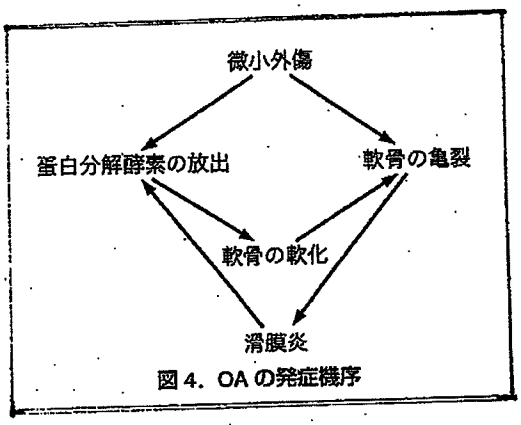


図4. OAの発症機序

関節軟骨は潤滑液が存在する限りは耐摩耗性に非常に優れていることが知られている[2,3]. 実際に関節の摩擦係数(μ)は0.002以下であると報告されている[4]. ちなみに, 氷とスケートのあいだの摩擦係数は0.01といわれている. さらに荷重が加わると, 関節軟骨層からより多量の組織液が関節面に滲出し, 摩擦係数は大きく変化しない仕組みになっている. この関節の潤滑は動的流体潤滑(hydrodynamic lubrication)で, その一部を説明することができる[5]. MacConaill[6]は, 非対称な関節における関節面の不適合性により関節の潤滑を説明している(図2). この動的流体潤滑はくさび膜潤滑ともいい, 滑液が摩擦面に引きずられて狭い隙間に向かって流動することにより, 圧力を生じ負荷を支えるものである. しかし現時点では, 生体関節の潤滑現象のすべてを単独で説明できる理論はなく, 種々の機構が関節運動の種類, 荷重の量などに応じて機能するものと考えられている.

関節面の相対する面の形状は完全ではない. もっとも適合性がよいと思われる股関節でも不適合性が報告されている[7]. しかし, このことが上述したように滑液の潤滑を可能にしている. さらに関節軟骨の存在は接触面の応力の集中を緩和するように働いている. すなわち, 関節の接触面積が狭いと接触面にかかる応力は集中し, 運動時にかかる剪断力も大きくなる. しかし, この場合に関節軟骨が存在していると, その弾性変形により図3に示すように応力集中が緩和され, 軟骨そのものに対する負荷や軟骨下骨に対する負荷も軽減する.

いままで述べてきたように, 関節軟骨の重要な2つの機能として荷重伝達と潤滑があげられる. 荷重に関与する因子としては保水性, 軟骨の塑性変形, 関節の形状などがあげられる. また潤滑に関しては, 関節液の流れ, 表面の粗さ, 表面の形状, 関節液の性状などがあげられる. これらが正常と異なればOAになると考えられるが, もっともはじめに起る変化としては軟骨の連続性の変化, 関節液の性状の変化, 患者の活動性の変化があげられる. これらの変化は関節の荷重や潤滑機構の破綻をきたし, 軟骨が変性する. 関節軟骨表面が徐々にすり減るか否かははっきりしていないが, 対向する関節面の動きにより関節軟骨に剪断力が働き, 圧縮力とともに軟骨変性の原因となる. これが加齢とともにすすむだけでなく, 機械的因子と関節内の生化学的変化と相俟って磨耗してくるものと思われる(図4). 具体的には, 軟骨の代謝が変化すると軟骨の細孔の大きさが変化し, 浸透圧がかわり軟骨基質が滲出し, 蛋白分解酵素により基質が変化する. OAの軟骨組織では, コラーゲン線維の網目構造の欠陥やプロテオグリカン巨大分子の損失により, 軟骨の透過性(permeability：その物質中の液体の流動に対する多孔性物質中の固形基質の摩擦抵抗を表す1つの物性のパラメータ)は大きくなる. したがって, 軟骨のクリープ変形が大きくなるため軟骨は力学的に不利な状態になる. OAでは軟骨細胞由来の分解酵素による内因性破壊に, 二次性の滑膜炎による滑膜や血球由来の分解酵素による外因性の破壊が加わる. 動物モデルによる研究

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of

Noboru YAMAJI, et al.

Appln. No.: 10/525,015

Confirmation No.: 5025

Filed: February 17, 2005

Docket No: Q86324

Group Art Unit: 1654

Examiner: Andrew D. KOSAR

For: AN AGENT FOR INHIBITING ARTICULAR CARTILAGE EXTRACELLULAR MATRIX DEGRADATION

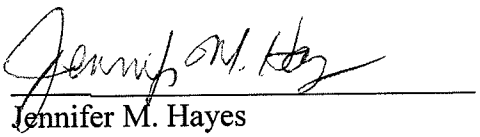
SUBMISSION OF APPEAL BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith please find an Appeal Brief. The USPTO is directed and authorized to charge the statutory fee of $540.00 and all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



Jennifer M. Hayes
Registration No. 40,641

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON DC SUGHRUE/265550

65565
CUSTOMER NUMBER

Date: October 3, 2008